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TITLE: Patterns of Proteins That Associate with p53 or with p53 Binding Sites Present in the Ribosomal Gene Cluster and MDM2(P2) Promoter

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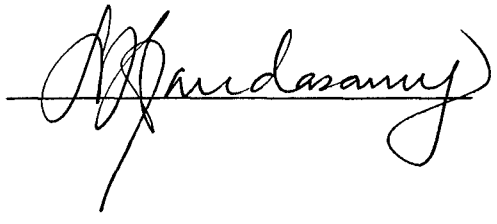

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13. ABSTRACT *(Maximum 200 words)*

P53 is a tumor suppressorr protein and its lack of function has been implicated in many human cancers. To understand the molecular mechanisms underlying p53 function, will certainly provide tools to develop more effective therapeutical approaches against cancer.

Our goal is to identify cellular proteins that associate wit wild-type p53 when p53 is bound to its binding site present in the internal promoter or the MDM2 gene (MDM2 P2) and when it is bound to its cognate site in the Ribosomal Gene Cluster.

Using DNA affinity chromatography, we have found that the TATA binding protein (TBP) and two of its associatee factors, TAFII40 and TAFII60, form a complex with wild-type p53 when p53 bids its cognate site in the MDM2 P2.

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Alfred Paul Dollo GS 8/30/99

PI - Signature Date

TABLE OF CONTENT.

INTRODUCTION.

The purpose of this project is to gain more understanding about the molecular mechanisms by which p53 exerts its function(s). Some of these mechanisms are already known. p53 is a sequence-specific DNA binding protein known to act as a transcription factor (reviewed by Prives, C. and Hall, P.A., 1999; El-Deiry W.S., 1998). *In vitro* and *in vivo* experiments have shown that p53 can activate transcription of some genes as well as suppress transcription for other DNA elements (Reviewed by El-Deiry, W.S., 1998). In addition, p53 can bind to DNA elements, for which no function has yet been identified, for example, The Ribosomal Gene Cluster (RGC) (Kern, S.E. et al.1991). To further understand the mechanisms underlying p53 function(s), our goal is to identify cellular proteins that interact with p53. Specifically, the wild type p53 present in the normal breast cell line MCF10A (Muller, F. R. et al. 1994), and in the breast cancer cell line ZR75-1 (Engel, W.L., et al. 1978). We also will analyze the proteins that interact with the mutant form of p53 Hist 273 present in the breast cancer cell line MDA-MB-468 (Bartek, et al. 1990; Nigro, J.M., et al 1989; Chen, J.Y. et al. 1993). This analysis will be done by DNA affinity purification using the p53 specific binding sites present in the Ribosomal Gene Cluster (RGC) (Kern, S.E. et al. 1991) and in the Promoter 2 (P2) of the MDM2 gene (Juven, T. et al. 1993). The binding of p53 to its cognate site present in the MDM2 (P2) promoter activates transcription of the MDM2 gene (reviewed by Prives, C. and Hall, P.A., 1999; El-Deiry W.S., 1998), while the function of the p53 binding site present in the RGC is not known. The p53 cellular level, under normal circumstances, is very low but upon DNA damage it is increased due to decreased degradation of the protein (Hall, P. A., et al. 1993). Because of this, and as a first control, we have set up the experimental conditions using the Ts. mutant p53 Val.135 present in the mouse fibroblast cell line 3-4. The Ts. mutant p53 Val. 135 provides a well-documented system for studying wild-type p53 dependent growth arrest, when the cells are shifted to 32 °C (Michalovitz, D. et al. 1990; Martinez, J. et al.1991).

In vitro and ***in vivo*** experiments have shown that p53 has the ability to associate with other proteins and that this association modulates p53 function (reviewed by Pives, C. and Hall, P.A., 1999). Most of these p53-associated proteins have been identified by co-immunoprecipitation experiments and using purified in vitro translated or fusion proteins. We think that the presence of the p53-binding site makes a difference regarding p53 association with other proteins in light of the fact that p53 changes its conformation when bound to DNA. Posttranslational modifications to p53 also influence p53 structure. To the best of our knowledge, this is the first time that p53-associated proteins are being studied by DNA affinity chromatography.

STATEMENT OF WORK.

Task 1. Define the experimental conditions for MDM2 affinity chromatography and for MDM2-GRAB as well as establish positive and negative controls for the MDM2-p53 associated proteins using nuclear extract from 3-4 and 10-1 cells respectively. Month 1-12.

Task 2. Define the experimental conditions for RGC affinity chromatography as well as establish positive and negative control for RGC-p53 associated protein using nuclear extract from 3-4 and 10-1 cells respectively. Month 13-15.

Task 3. Extrapolate results from tasks 1 and 2 to a normal breast cell line by performing MDM2 and RGC affinity chromatography with nuclear extract from MDA10A cells. Month 16-18.

Task 4. Compare the results from a normal breast cell line before and after p53 activation by performing MDM2 and RGC affinity chromatography with nuclear extract from MDA10A cells before and after treatment with Actinomycin as well as from elutriated cells and comparing with results from task 3. Month 19-21.

Task 5. Extrapolate the results from a normal breast cell line without activating p53 to the results from a breast cancer cell line that has wild-type p53 by performing MDM2 and RGC affinity chromatography with nuclear extract from ZR75-1 cells and comparing these results to those from task 3. Month 22-24.

Task 6. Compare the results from a breast cancer cell line that has wild-type p53 before and after p53 activation by performing MDM2 and RGC affinity chromatography with nuclear extract from ZR75-1 cells before and after treatment with Actinomycin as well as from elutriated cells and comparing with results from task 5. Month 25-27.

Task 7. Extrapolate the results from a breast cancer cell line that has wild-type p53, to the results from a breast cancer cell line that has the mutant p53 His 273. This will be done by performing MDM2 and RGC affinity chromatography with nuclear extract from MDAMB468 cells and comparing these results to those from task 5. Month 28-30.

Task 8. Compare the results from a breast cancer cell line that has the mutant p53 His 273, before and after p53 activation. This will be done by performing MDM2 and RGC affinity chromatography with nuclear extract from MDAMB468 cells before and after treatment with Actinomycin as well as from elutriated cells and comparing with results from task 7. Month 31-33.

Task 9. Extrapolate the results from a breast cancer cell line lacking p53 to the result s from breast cancer cell lines with wild-type and mutant Hist 273 p53 by performing the experiments with nuclear extract from MDAMB157 cells (before and after Actinomycin treatment and elutriation) and comparing them to results from tasks 5, 6, 7and 8 Month 34-36.

RESULTS.

Task 1. Define the experimental conditions for MDM2 affinity chromatography and for MDM2-GRAB as well as establish positive and negative controls for the MDM2-p53 associated proteins using nuclear extract from 3-4 and 10-1 cells respectively. Month 1-12.

Most of task 1 was developed during the first year and the results were presented in the report for 1998. That report shows that p53 can be successfully purified by DNA affinity chromatography using the p53 binding sites present in The Ribosomal Gene Cluster (RGC) and in the internal promoter of the MDM2 gene (MDM2 P2). It was also shown in that report that besides p53, another protein (or protein complex) eluted from the MDM2 P2 column. This protein (or protein complex) was able to bind to a deoxyoligonucleotide corresponding to a TATA Box in Electrophoretic mobility Shift experiments. Because of its ability to bind to a TATA Box, it was assumed that TBP was part of this protein complex. In order to confirm the presence of TBP in the MDM2 P2 elution fractions and its association with p53, 10 ug of p53 contained in the nuclear extract from 3-4 cells was loaded onto the MDM2 P2 affinity column and DNA affinity chromatography was performed as described in methods. As a negative control, a parallel experiment was performed using equivalent amount of total protein contained in the cell extract from the 10-1 cells (no p53).

P53 is present in the elution fractions from the MDM2 P2 affinity column. In order to determine the presence of p53 in the elution fractions from the MDM2 P2 column, an Electrophorectic Mobility Shift assay was carried out as described in methods. 5% of each elution fraction was incubated with the p53 Super Consensus Sequence (SCS); 421 anti- p53 antibody was used, were specified, to activate p53 binding. In the case of the 3-4 cell extract, a small amount of p53 eluted at fractions 0.3 and 0.4 molar KCl (fig 1A, lanes 11 and 12). Only a small fraction of the total p53 loaded onto the column (10 ug) was bound to it (in the order of nanograms). This fact was not due to a saturation of the column with p53 given that the same column was able to bind a greater amount of a control p53 preparation in a subsequent experiment (data not shown). Besides the band corresponding to p53, another band was evident. In previous experiments, presented in the 1998 report, a band was present when the elution fractions were incubated with a TATA box. Because the ability of this protein to bind to a TATA box we propose that this unknown complex may contain TBP (fig. 1A, lanes 11 to 17). In the other hand, the MDM2 P2 elution fractions of the 10-1 cell extract showed no p53 band, as expected (fig 1B). Interestingly, the unknown complex band was present in these fractions (Fig. 1B, lanes 12 to15).

TBP is present in the MDM2 P2 elution fractions. Western Blot and inmunoprobing techniques were used to determine the presence of TBP in the elution fractions from the MDM2 P2 affinity column. 80% of each elution fraction was used for western blot analysis as described in methods. The nitrocellulose membrane was probed with anti TBP antibody (Santa Cruz) (fig. 2). TBP, present in the 3-4 cells, eluted at 0.4 to 0.9 molar KCl (fig. 2A, lanes 3 to 8) while the TBP present in the 10-1 cells eluted at 0.3 to 0.7 molar KCl (fig. 2B, lanes2 to 6). This correlated with the fractions showing the unknown complex in the EMSAs.

P53 from the MDM2 P2 column binds to TBP complex-TATA box. 5% of each elution fraction from the MDM2 P2 affinity column was incubated with TBP consensus sequence and an Electrophoretic Mobility Shift assay was carried out as described in methods (Fig.3). In the elution fractions from the MDM2 P2 column, a supershift of the TBP complex band was evident when p53 was present in the nuclear extract (3-4 cells) (fig. 3A, lanes 7,8). The supershift of the TBP complex band was not observed in the case of the MDM2 P2 elution fractions from the 10-1 cell extract (no p53) (fig. 3B).

TBP is present in the TBP complex band. In order to corroborate the presence of TBP in the TBP complex band, an Electrophoretic Mobility Shift assay was performed as described in methods. 5% of fraction 0.3 molar KCl from the MDM2 P2 column was incubated with TBP consensus sequence (Santa Cruz); anti TBP (generous gift of Roeder Laboratory) was included in the reaction as indicated (Fig 4). 2ul of the anti TBP preparation produced a supershift of the TBP complex band (fig.4, lane 2) as compared with the control (fig. 4,

lane 4). When the amount of anti TBP antibody was increased to 6 ul, there was a corresponding increase in the amount of the supershift observed (fig. 4, lane 3).

TAFII40 and TAFII60 are members of TBP complex. In order to determine the presence of TAFII40 and TAFII60 in the TBP complex band, Electrophoretic Mobility Shift assay was performed as described in methods. 5% of fraction 0.3 molar KCl from the MDM2 P2 column was incubated with TFIID consensus sequence (Santa Cruz); anti TAFII40 and anti TAFII60 antibodies (Roeder Laboratory) were included in the reaction as indicated. (Fig 5). 6ul of each antibody preparation produced a supershift of the TBP complex band (fig.5, lane 3) for TAFII40 and (fig. 5, lane 4) for TAFII60 as compared with the control (fig. 5, lane 1) which has no antibody or (fig. 5, lane 2) where a non relevant antibody was added.

Task 2. Define the experimental conditions for RGC affinity chromatography as well as establish positive and negative control for RGC-p53 associated protein using nuclear extract from 3-4 and 10-1 cells respectively. Month 13-15.

In order to study the binding of p53 to the p53 binding site present in the Ribosomal Gene Cluster (RGC), 10 ug of p53 contained in the nuclear extract from 3-4 cells was loaded onto the RGC affinity column and DNA affinity chromatography was performed as described in methods. As a negative control, a parallel experiment was performed using equivalent amount of total protein contained in the cell extract from the 10-1 cells (no p53).

Binding of p53 to the RGC affinity column. In order to determine the presence of p53 in the elution fractions from the RGC affinity column, an Electrophorectic Mobility Shift assay was carried out as described in methods. 5% of each elution fraction was incubated with p53 Super Consensus Sequence (SCS); 421 anti p53 antibody was used, were specified, to activate p53 binding. Our results showed a band that migrates as p53 and that eluted at 0.3 and 0.4 molar KCl (fig 6A, lanes 11 and 12) but which was not supershifted by the monoclonal antibody 421. Because this protein binds to a p53 super consensus binding site and because is not present in the RGC elution fraction from the 10-1 cells (fig. 6B), it is very likely to correspond to p53. This fact has to be confirmed by further experiments. Besides the band corresponding to p53, the band corresponding to unknown/TBP complex was also present in the RGC elution fractions from the 3-4 cells (fig. 6A, lanes 12 to 18) as well as in the elution fractions from the 10-1 cells (fig. 6B, lanes 13 to 18).

TBP complex binds to RGC. In order to confirm the presence of the TBP complex in the elution fractions from the RGC affinity column, 5% of each elution fraction was incubated with TFIID consensus sequence (Santa Cruz) and EMSA experiments were conducted as specified in methods. The TBP complex was present in the RGC elution fractions from the 3-4 cells and eluted at 0.4 to 0.8 molar KCl (fig.7A, lanes 12 to 16). The TBP complex was also present in the RGC elution fractions from the 10-1 cells, in this case it eluted at 0.5 to 0.8 molar KCl (fig. 7B, lanes 11 to 14).

P53 from RGC does not bind to the TBP complex. The presence of p53 (3-4 cells) in the RGC elution fractions did not produce a supershift of the TBP complex as it did in the case of the MDM2 P2 elution fractions; compare fig. 7A, lanes 12 to 14 with fig. 3A, lanes 7 and 8.

DISCUSSION.

Our results showed that p53 from the MDM2 P2 column associated with TBP complex (fig.3). p53 from the RGC site did not associate with TBP complex (fig. 7). The TBP complex band corresponds to a complex formed, at least, by the TATA Binding Protein (TBP) and two of its associated factors TAFII40 and TAFII60 (Fig. 4 and 5); the presence of other proteins in this complex remains to be studied. The TBP complex eluted from the MDM2 P2 (fig. 1and 3) and RGC (fig. 6 and 7) affinity columns and the unknown complex bound the

Super Consensus Sequence and the TFIID oligos in a similar way and in a p53 independent manner. This fact might be due to the ability of TBP to bind DNA in both specific and non-specific way.

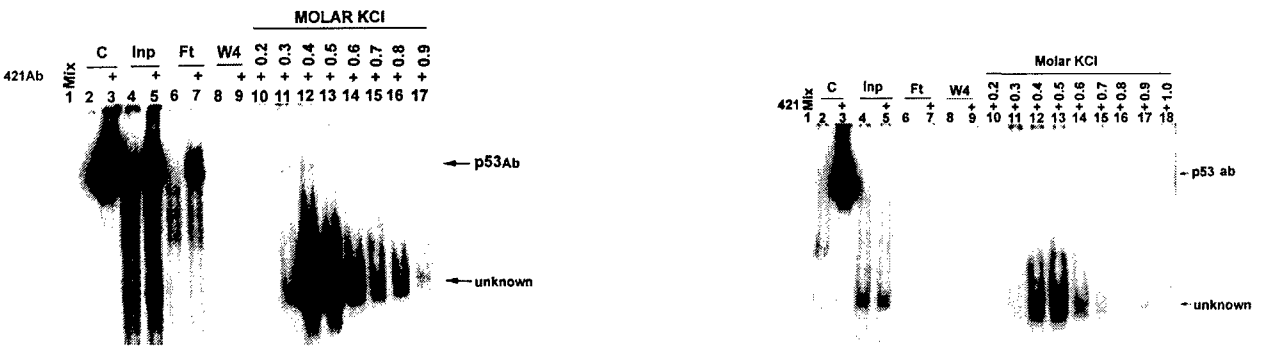
The fact that not all the loaded p53 binds to the column might indicate that each p53 binding site selects for a specific subtype of p53. This seems to be supported by the fact that the putative p53 eluted from the RGC affinity column was not shifted by the monoclonal antibody 421 (Fig. 6A) while the p53 that binds the MDM2 P2 column responds to this antibody (fig. 1A). If this turns out to be the case, DNA affinity chromatography is a suitable technique to further analyze biochemical features of different p53 subtypes, despite the fact that further experiments are needed to confirm the identity of the putative p53 band present in the RGC elution fractions.

The experiments corresponding to tasks 3,4 and 5 could not be accomplished as planned due to technical difficulties. One of the main challenges of this project has been to elute, from the columns, enough sample to perform the identification steps. Our initial plan was to identify the associated protein by EMSA experiments as well as by western blot and immunodetection. In an attempt to have enough sample to perform the immunodetection experiments, we scaled up the procedure as much as we could. This scaling up implied the usage of an enormous amount of cells and cell extracts, which made each step much longer. In the future, we will identify the proteins by EMSA assay, which has proven to be accurate and suitable for our experimental system.

FIGURES.

FIGURE 1

A ***B***

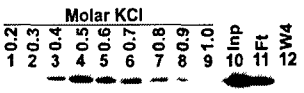


P53 is present in the elution fractions from the MDM2 P2 affinity column.

A. 10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cells nuclear extract, were loaded onto the MDM2 P2 affinity column and DNA affinity chromatography was performed as specified in methods.. The elution fractions were analyzed by EMSA assay. 5% of each elution fraction was incubated with a ^{32}p labeled deoxyoligonucleotide corresponding to a p53 binding site (SCS). ***B****. 7 mg. of total protein (normalized to experiment A) contained in the Sephacryl S300 fraction pool from 10-1 cells nuclear extract were loaded onto a MDM2 P2 affinity column and the experiment was conducted as described in part A.*

IGURE 2.

A.



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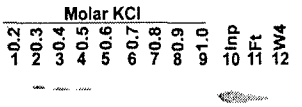
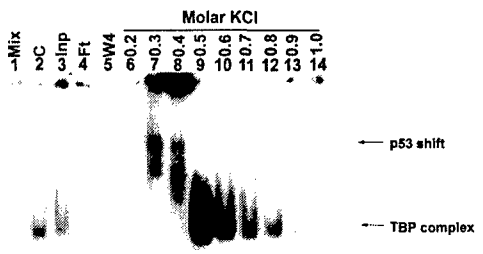


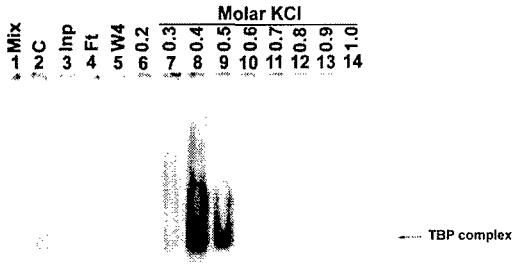
TBP is present in the MDM2 P2 elution fractions. *80% of each elution fraction from the MDM2 P2 column was resolved by SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti TBP antibody (Santa Cruz). A. 3-4 cell extract. B. 10-1 cell extract.*

FIGURE 3.

A



B



P53 from the MDM2 P2 column binds to TBP complex-TATA box. *A.10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cells nuclear extract, were loaded onto the MDM2 P2 affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by EMSA assay, as described in methods. 5% of each elution fraction was incubated with a ^{32}p labeled deoxyoligonucleotide corresponding to a TATA box (TFIID consensus oligo, Santa Cruz). B. 7 mg. of total protein (normalized to experiment A) contained in the Sephacryl S300 fraction pool from 10-1 cells nuclear extract were loaded onto a MDM2 P2 affinity column and the experiment was conducted as described in part A.*

.FIGURE 4.

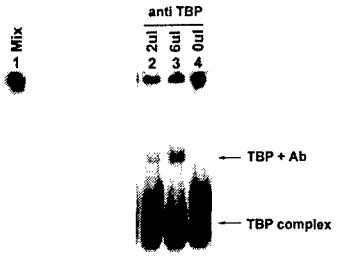
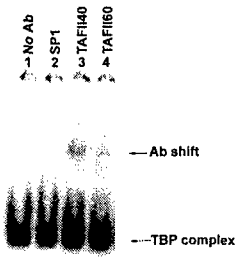


FIGURE 5.



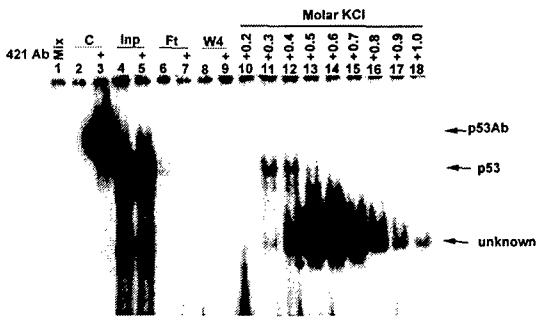
TBP is present in TBP complex band. *5% of elution fraction 0.3 molar KCl from the MDM2 P2 column and 3-4 cell extract was incubated with ^{32}p labeled TFIID consensus sequence (Santa Cruz). Different amounts a polyclonal anti TBP antibody preparation (Roeder laboratory) were included in the reaction mixture.*

FIGURE 5.

TAFII40 and TAFII60 are members of TBP complex. *5% of elution fraction 0.3 molar KCl from the MDM2 P2 column and 3-4 cell extract was incubated with ^{32}p labeled TFIID consensus sequence (Santa Cruz). Different amounts a polyclonal anti TAFII40 and anti TAFII60 antibody preparation (Roeder laboratory) were included in the reaction mixture specified in figure.*

FIGURE 6.

A.



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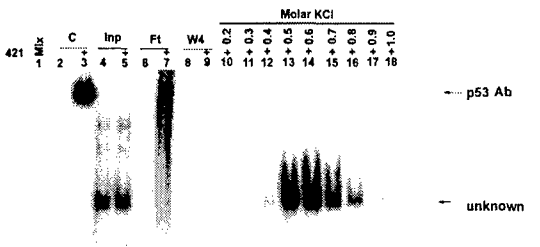
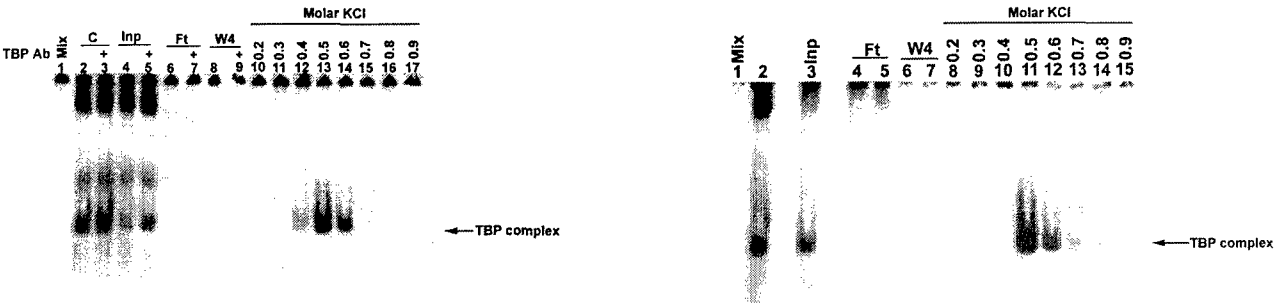


Binding of p53 to the RGC affinity column. ***A.****10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cells nuclear extract, were loaded onto the RGC affinity column and DNA affinity chromatography was performed as specified in methods.. The elution fractions were analyzed by EMSA assay as described in methods. 5% of each elution fraction was incubated with a ^{32}p labeled deoxyoligonucleotide corresponding to a p53 binding site (SCS).* ***B.*** *7 mg. of total protein (normalized to experiment A) contained in the Sephacryl S300 fraction pool from 10-1 cells nuclear extract were loaded onto a RGC affinity column and the experiment was conducted as described in part A.*

FIGURE 7.



P53 from RGC does no binds to TBP complex. *10 ug of p53, contained in the Sephacryl S300 fraction pool prepared from 3-4 cells nuclear extract, were loaded onto the RGC affinity column and DNA affinity chromatography was performed as specified in methods. The elution fractions were analyzed by EMSA assay, as described in methods. 5% of each elution fraction was incubated with a ^{32}p labeled deoxyoligonucleotide corresponding to a TATA box (TFIID consensus oligo, Santa Cruz). B. 7 mg. of total protein (normalized to experiment A) contained in the Sephacryl S300 fraction pool from 10-1 cells nuclear extract were loaded onto the RGC affinity column and the experiment was conducted as described in part A.*

FINDINGS.

- The form of p53 that binds the p53 binding site present in the internal promoter of the of the MDM2 gene (MDM2 P2) forms a complex with the TATA Binding Protein TBP and two of its associated factors, TAFII40 and TAFII60.

- DNA affinity Chromatography is a suitable technique to further study of the biochemical properties of p53.

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 17 Oct 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for grants. Request the limited distribution statements for the Accession Document Numbers listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Enclosure